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Juvenile hormone regulation of *Drosophila Epac*—A guanine nucleotide exchange factor

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ABSTRACT

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to characterize the effects of juvenile hormone (JH) on *Epac* (*E*xchange *P*rotein directly *A*ctivated by *C*yclic AMP; NM_001103732), a guanine nucleotide exchange factor for Rap1 in *Drosophila* S2 cells. JH treatment led to a rapid, dose-dependent increase in *Epac* relative expression ratio (RER) when compared to treatment with methyl linoleate (MLA) that lacks biological activity. The minimal level of hormone needed to elicit a response was 100 ng/ml. Time-course studies indicated a significant rise in the RER 1 h after treatment. S2 cells were challenged with 20-hydroxyecdysone and a series of compounds similar in structure to JH to determine the specificity of the response. Methoprene and JH III displayed the greatest increases in RER. Late third instar (96 h) *Drosophila* were exposed to diet containing methoprene (500 ng/g diet); significantly higher RERs for *Epac* were observed 12 h after exposure. JH had no effect on *Epac* RERs in the human cell line HEK-293.

1. Introduction

The insect juvenile hormones (JHs) represent a family of acyclic sesquiterpenoids that regulate a diversity of processes in the insect life cycle (Nijhout, 1994; Riddiford, 1996; Goodman and Granger, 2005; Jones and Jones, 2007; Truman and Riddiford, 2007). JH affects insect development by maintaining the larval stage and inhibiting metamorphosis. In adults, JH is involved in regulating reproductive physiology (Riddiford, 1996; Wilson et al., 2003; Belles, 2004; Flatt et al., 2005). Although well-studied from the physiological standpoint, the molecular mechanisms underlying JH action during larval development and metamorphosis remain largely unknown (Jones, 1995; Kethidi et al., 2006; Riddiford, 2008).

Attempts to develop a unifying concept regarding the mode of action of JH, encompassing a multitude of highly complex model systems and various insect species, has proven difficult (see Jones and Jones, 2007; Riddiford, 2008 for reviews). Capitalizing on the wealth of information derived from the molecular action of vertebrate hormones, two potential modes of action for JH have been suggested: activation of nuclear elements via transcription factors and activation of signal transduction pathways. The lipophilic nature of JH and its potential transit through the plasma membrane has led some investigators to suggest that JH acts through a specific nuclear receptor complex that modulates gene expression at the level of transcription (Shemshedini et al., 1990; Riddiford, 1996; Jones and Sharp, 1997; Wilson et al., 2003; Jones and Jones, 2007; Riddiford, 2008). Indeed, there are a series of reports that suggest embryonic and larval cells display high-affinity binding proteins for JH (Chang et al., 1980; Klages et al., 1980; Wisniewski and Kochman, 1984; Wisniewski et al., 1988; Palli et al., 1990); however, the links between these hormone-interacting proteins and transcription processes are unclear.

There is mounting evidence that JH may act at the membrane level, triggering the release of receptor-mediated signal transducing molecules. These studies were conducted in adult reproductive tissues (Yamamoto et al., 1988; Sevala and Davey, 1989; Pszczolkowski et al., 2005); however, one recent report examined the role of JH in the regulation of protein kinase C activity in *Drosophila* L57 cells (Kethidi et al., 2006) that were originally derived from early-stage embryos (Echalier, 1997). Recently, we examined the global response of *Drosophila* S2 cells to a JH challenge using a microchip array and subsequent validation using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Willis et al., in press). Using methyl linoleate (MLA) to factor out nonspecific lipid effects, the transcript abundance from 13 known or putative genes increased significantly in the presence of JH. We

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confirmed the change in relative expression ratio (RER) of a subset of these loci by real-time qRT-PCR using three validated reference transcripts that exhibited constant expression in the presence or absence of JH III. One of the most interesting genes to be identified in the study was *E*xchange *P*rotein directly *A*ctivated by *C*yclic AMP (*Epac*), a guanine nucleotide exchange factor (GEF) for Rap1 small GTPase. In the presence of JH, the RER of *Epac* increased nearly 4-fold over a 4-h period (Willis et al., in press).

While early studies ascribed the activation of protein kinase A (PKA) by cyclic AMP (cAMP) as the primary link between heterotrimeric G-protein-coupled receptors and their targets, more recent evidence suggests that Epac also plays an important role (Bos, 2006). Epac, upon activation by cAMP, aides Rap1 as it cycles between an inactive GDP-bound and an active GTP-bound conformation (de Rooij et al., 1998). Rap1 activity is modulated by several GEFs including PDZ, C3G, DOCK4, RapGRP, and Epac (Bos, 2005). In turn, Rap1 modifies cell adhesion processes through cadherin-mediated cell junction formation and integrin-mediated cell adhesion (Bos, 2005). In studies on *Drosophila* development, Asha et al. (1999) demonstrated that Rap1 plays a critical role in regulating normal morphogenesis in eye discs, ovaries and embryos. In addition, Rap1 mutations disrupt cell migration and induce abnormalities in cell shape, further implicating Rap proteins as regulators of morphogenesis in vivo. While the morphogenetic role of JH in maintaining the larval state has been recognized for some time (see Goodman and Granger, 2005; Jones and Jones, 2007 for reviews), molecular targets for the hormone that reside in known signal pathways are lacking. In this work, we characterize the dose–response and specificity of *Epac* induction by JH and report that JH increases the RER of *Epac*, a key component in a PKA-independent pathway that targets Rap1, a critical molecule in morphogenesis.

2. Materials and methods

2.1. Purification and quantification of JH homologs, agonists, and 20-hydroxyecdysone

Racemic JH I was purchased from Scitech (Prague) while JH III, MLA and methyl linolelaidate (MLEA) were purchased from Sigma Chemicals (St. Louis). Bisepoxy JH III (JHB_3) was a gift from Prof. L.I. Gilbert (University of North Carolina, Chapel Hill). (*S*) Methoprene was obtained from Hartz Mountain Corp (Secaucus, NJ) in the form of tick and flea control capsules. 20-Hydroxyecdysone (20E) was obtained from Mann Research (New York). 3-Octylthio-1,1,1,-trifluoropropan-2-one (OTFP), a potent JH esterase inhibitor, was a gift from Prof. Y.C. Toong, Universiti Sains, Malaysia.

The biologically active enantiomers (10*R*,11*S*) JH I, (10*R*) JH III and (10*R*) JHB_3 were isolated from racemic mixtures by chiral HPLC chromatography (Cusson et al., 1997). (*S*) Methoprene was extracted from flea control capsules using acetone. The acetone extract containing methoprene was then developed on normal phase TLC plates (Silica gel 60 F-254; E. Merck, Darmstadt) using a toluene:ethyl acetate:acetic acid (100:30:3, v/v/v) mobile phase as modified from Schooley et al. (1975). The only band detected was scraped from the plate and extracted using ethyl acetate. HPLC analysis of the extract (hexane:diethyl ether, 96:4, v:v; 5 μm, Spherosorb silica column) indicated the methoprene to be >95% pure as determined by UV spectroscopy at 230 nm. The mass of JH and JH acid was determined by UV spectrophotometry (Goodman et al., 1978; Goodman and Adams, 1984). Synthesis and purification of (10*R*) JH III acid was performed using the procedure outlined by Goodman and Adams (1984). Due to the instability of JH acid, it was used within 1 week of its preparation from JH III. Hormone stability in culture medium was analyzed using [^{3}H]-JH III (PerkinElmer, Boston, MA). Radiolabeled JH III was added to the medium and then recovered by phase separation (ethyl acetate:medium, 2:1, v/v). The ethyl acetate phase was dried under a gentle stream of N_2 and the extract developed on a normal phase TLC plate (hexane:ethyl acetate, 85:15, v/v). Areas containing JH and JH metabolites were recovered and radio assayed.

2.2. Preparation of charcoal dextran-stripped bovine serum albumin and medium

To remove potential contaminants, bovine serum albumin (BSA; Pentex Fraction V, Miles Diagnostics, Kankakee, IL) was stripped using activated charcoal. Charcoal (1.25 g; Sigma) was added to 500 ml of Tris–HCl (0.1 M, pH 8.0) containing 0.25 g dextran (T500; Sigma). After stirring overnight at 4 °C, 100 ml of this suspension was centrifuged at 1000 × *g* for 10 min. The dextran-coated charcoal pellet was mixed with 100 ml 10% BSA (in phosphate buffered saline, pH 7.4) and incubated at 45 °C for 45 min. Charcoal was then removed by centrifugation and the BSA was sterilized by passage through a 0.22-μm filter (Millipore, Billerica, MA).

Since S2 cells display JH esterase activity (Dubrovsky et al., 2004), OTFP (Venkatesh et al., 1990) was added to the medium at a final concentration of 1 μM and shaken at 4 °C for 2 h before adding the potential agonists or controls. Hormones and agonists were incubated with the charcoal-stripped BSA overnight at 4 °C and then added to the medium containing OTFP to produce a final concentration of 1% BSA.

2.3. Cell culture

Drosophila S2 cells (Invitrogen, Carlsbad, CA) were maintained in SF900 serum-free medium (Invitrogen) at 27 °C. Cells (5×10^5/ml) were seeded into 60 mm × 15 mm plastic dishes (Nunc, Roskilde, Denmark) containing 3 ml of medium and grown for 36 h. Cells at approximately 80% confluence were then challenged with the test compounds. Control cells were treated with 1% BSA alone or 1% BSA with 250 ng/ml MLA.

Human embryonic kidney cells (HEK-293, ATCC, Manassas, VA) were cultured in DMEM medium (ATCC) with 2 mM L-glutamine and 10% fetal bovine serum at 37 °C, 5% CO_2. Cells (4×10^5/well) were seeded into 6-well plates containing 2 ml medium. At 60% confluence, they were treated with 250 ng/ml hormone. The human β-actin gene (GenBank Accession Number: NM_001101) was used as an internal reference standard.

2.4. Drosophila rearing and staging

Canton-S wild type *Drosophila melanogaster* (provided by Prof. C. Cirelli, University of Wisconsin-Madison) were raised at 25 °C using a 12:12 L:D photocycle and maintained on Fisher instant *Drosophila* diet seeded with active dry yeast. Larvae used in these experiments were staged by collecting eggs oviposited during a 2-h period. These insects were allowed to develop in untreated diet until the mid-third stadium (96 ± 2 h). At 96 h, insects were transferred to a 60 mm × 15 mm plastic dish containing either agonist or control diet. Diet containing methoprene (500 ng/g diet) was prepared by dissolving the agonist in ethanol, applying it to the surface of the medium and allowing the ethanol to evaporate. After evaporation, the diet was thoroughly mixed to distribute the agonist and 5 g placed into 60 mm × 15 mm plastic dishes. Control diet was similarity prepared using ethanol only. Fifteen mid-third instars were placed in each of three dishes for each treatment and allowed to feed for 6, 12 or 18 h. At each time point and for each treatment, 10 larvae from each dish were collected, rapidly frozen in liquid nitrogen and stored at −80 °C until processed.

2.5. Isolation of RNA and cDNA synthesis

Cells were lysed directly in the culture dishes and total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was exhaustively treated with 2 U of Turbo DNase (Ambion, Austin, TX) for 1 h at 37 °C and quantified by UV spectrophotometry using a NanoDrop ND 1000 spectrophotometer. Absence of DNA contamination in total RNA was confirmed by real-time qRT-PCR as previously described (Rotenberg et al., 2006; Jahn et al., 2008; Willis et al., in press). RNA quality and integrity was determined by gel electrophoresis on standard agarose gels or capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). First-strand cDNA synthesis was performed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) as previously described (Willis et al., in press).

2.6. Extraction of RNA from whole body

Larvae were homogenized in lysis buffer (RNeasy Mini Kit) using a pellet pestle (Kimble-Kontes, Vineland, NJ) and QIA shredder columns (Qiagen). Total RNA was extracted as described by the manufacturer for animal tissue. Larval RNA was processed as described above.

2.7. Real-time qRT-PCR

Based on previous microarray analyses (Willis et al., in press), three gene transcripts unaffected by the addition of JH (GenBank Accession Numbers: NM_137495, NM_130639, and NM_170461) were selected as internal reference genes. Gene-specific primers were designed with Beacon Designer software (Premier Biosoft International, Palo Alto, CA). Amplification efficiency for all primer sets was optimized (Rotenberg et al., 2006; Jahn et al., 2008; Willis et al., in press) and their primer products were sequenced to insure accuracy. Expression of *Epac* mRNA was analyzed by real-time qRT-PCR using the Biorad iCycler iQ detection system (Bio-Rad Laboratories). *Epac* expression was normalized to the expression of the three validated reference gene transcripts. A thorough description of the real-time procedure and data analysis is described elsewhere (Rotenberg et al., 2006; Jahn et al., 2008; Willis et al., in press). Primer sequences and efficiencies are shown in Table 1.

2.8. Heterogeneous RNA analysis

Analysis of the abundance of *Epac* primary transcript or heterogeneous RNA (hnRNA) reflects transcription rates more reliably than the analysis of mRNA because of its short half-life (Elferink and Reiners, 1996). In the present study, one PCR primer

Table 1
Primers for real-time qRT-PCR.

Gene	Primer sequence (forward/reverse)[a]	Amplification efficiency[b]
Epac Guanine Nucleotide Exchange Factor NM_001103732	$_{nt\ 1152}$ CTTACTCAAGGGATCGGTAGAC/$_{nt\ 1284}$ CTCCTTCAGTACGATGGTAGC	1.97
Ribosomal protein rp49/RpL32 NM_170461	$_{nt\ 58}$ CCAAGATCGTGAAGAAGCG/$_{nt\ 197}$ GTTGGGCATCAGATACTGTC	1.99
Glutathione transferase NM_137495	$_{nt\ 47}$ GAGAAACTGCTTGTACTATGTC/$_{nt\ 206}$ AGAAACTCGGCGGATTTG	2.00
ATP binding protein NM_130639	$_{nt\ 1250}$ CATTGGAAGAAGCATTGGC/$_{nt\ 1413}$ CATAGTGGCGGATTCATCG	1.98
Cytochrome P450-6a2 NM_078904	$_{nt\ 379}$ TCAACCTGGACGGAAAGAAG/$_{nt\ 492}$ CGAACTCCTCAGACACCTTG	1.85
Epac Intron/Exon NT_033788	$_{nt\ 2650742}$ CACCCATTCCAGCCAAAGAC/$_{nt\ 2650642}$ CCATTACCACAGCGAAGAAGG	1.99
Human Epac1 NM_006105	$_{nt\ 1964}$ ATGAGCGTCTCTTTGTTG/$_{nt\ 2133}$ CTGGTGGATACTGTTGAAG	1.97
Human β-actin NM_001101.2	$_{nt\ 1466}$ GCATTGTTACAGGAAGTC/$_{nt\ 1591}$ TTACATAATTTACACGAAAGC	1.92

[a] The number of the nucleotide where the primer is located is designated by$_{nt}$.
[b] Primer efficiencies were determined from a serial dilution of target DNA using the formula $E = 10(-1/\text{slope})$ (Pfaffl et al., 2004 and Rasmussen, 2001), with the slope determined by the iCycler iQ software.

was designed to correspond to a region in exon 16 while the reverse primer was designed to correspond to a region in intron 15 (see Table 1).

3. Results

3.1. Introduction of agonists in vitro

Introduction of lipophilic agonists into an in vitro system devoid of serum poses a dispersal problem. JH and its agonists are surface-active and bind nonspecifically to hydrophobic surfaces (Kramer et al., 1976; Giese et al., 1977). To counter this problem, we used BSA as a carrier to reduce nonspecific binding. Since the BSA concentration may affect how the cells respond to JH, levels of both BSA and JH were varied so that the cells were exposed to 100–500 ng/ml JH in the presence of 0.1% or 1.0% BSA. Fig. 1 indicates that the RER of JH-stimulated *Epac* was nearly doubled when the concentration of BSA was increased from 0.1% to 1.0%. In addition to the problem of nonspecific lipid adherence, S2 cells express JH esterase activity (Dubrovsky et al., 2004) that rapidly metabolizes JH. Without the JH esterase inhibitor OTFP, better than 70% of the hormone was metabolized during the first hour of incubation in our system. This metabolism was significantly reduced when OTFP was present (data not shown).

3.2. Verification of JH induction of Epac under optimized conditions

Our initial work with *Drosophila* S2 cells using Affymetrix (Santa Clara, CA) microarray chips demonstrated that (10*R*) JH III induced a rapid (4 h) 3-fold increase in *Epac* (Willis et al., in press). In that study, JH and MLA were introduced to the culture medium in 0.1% BSA. To verify the JH increase of RER of *Epac* under more optimized conditions, *Drosophila* S2 cells were treated with (10*R*) JH III (250 ng/ml), MLA (250 ng/ml) or methoprene (250 ng/ml) in 1.0% BSA for 4 h and analyzed for RER of *Epac* using real-time qRT-PCR. Three reference genes, whose transcript abundance remained constant during the experimental period, were identified in preliminary microarray analyses (Willis et al., in press). They are NM_130639, whose gene product has ATP binding activity and is involved in response to stress; NM_137495, whose gene product is a glutathione transferase involved in a toxin defense response; and NM_170461, whose gene product is ribosomal protein 49. The ribosomal protein 49 gene is one of the most commonly employed to normalize gene expression in *Drosophila*. The mean RER of *Epac*, normalized to each of the three internal reference genes, verified that JH III increased the RER of *Epac* approximately 3-fold relative to the MLA control (Fig. 2). Mean *Epac* RERs were not statistically different between the controls 1.0% BSA and 1.0% BSA + MLA.

3.3. Intron–exon boundary studies

Since hnRNA is highly transient, its presence in the target cell is due, in part, to new transcription and splicing. In low-abundance genes such as *Epac*, real-time qRT-PCR monitoring of regions that span both an exon and an intron provide additional evidence that new transcription is occurring (Lipson and Baserga, 1989; Elferink and Reiners, 1996; Delany, 2001). Employing this method, we generated primers to a region between intron 15 and exon 16. Table 2

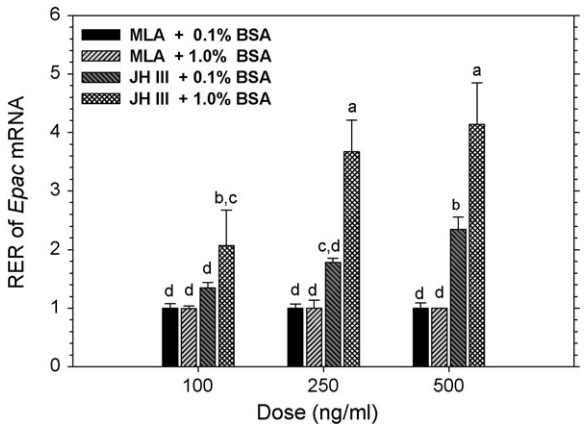

Fig. 1. Determination of the working concentration of BSA. S2 cells (5×10^5/ml) were seeded into 60 mm × 15 mm dishes containing 3 ml of medium and allowed to grow for 36 h. Cells were then challenged for 4 h with (10*R*) JH III or MLA in the presence of 0.1% or 1.0% BSA. Total RNA was extracted and the RER for *Epac* was determined by real-time qRT-PCR using MLA as a control. *Rp49* (FBGN0002626) was used as an internal standard reference gene. Error bars represent standard deviation ($n = 3$).

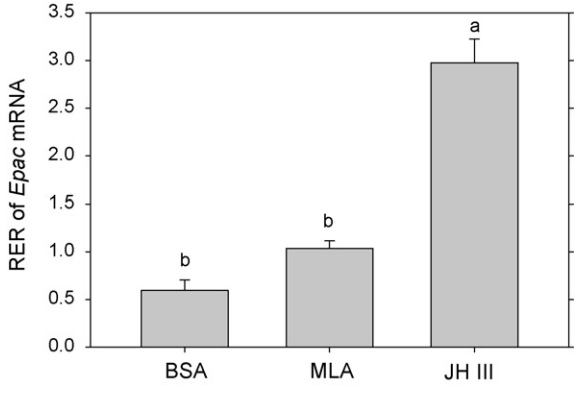

Fig. 2. Quantification of *Epac* induction by JH using three reference gene transcripts. S2 cells (5×10^5/ml) were seeded into 60 mm × 15 mm dishes containing 3 ml of medium and allowed to grow for 36 h. Cells were then challenged with 1% BSA, 1% BSA containing 250 ng/ml MLA, or 1% BSA containing 250 ng/ml (10*R*) JH III. Reference gene transcripts included FBGN0002626, *Rp49*; FBGN0034354, and FBGN0023529. Sequence of primer sets is listed in Table 1. Total RNA was extracted and the mean RER of *Epac* was determined by real-time qRT-PCR compared to MLA as the control. Error bars represent standard deviation ($n = 3$).

Table 2
Relative expression ratios of *Epac* hnRNA.

JH III	1.69 ± 0.25*
MLA	1.00 ± 0.59

RERs are the mean of three determinations; NM_170461, NM_137495, and NM_130639 were used as reference genes.

* Mean RER of *Epac* with JH III treatment is significantly different from MLA control as compared by one-way ANOVA (LSD; $p < 0.05$).

demonstrates a small but significant increase in the RER for *Epac* hnRNA when challenged with JH as compared to control.

3.4. Dose–response and time-course studies

To determine the concentration dependence of *Epac* expression, S2 cells were treated for 4 h with varying doses of (10*R*) JH III (1–500 ng/ml). The resulting data indicated that the RER of *Epac* mRNA levels rose in a dose-dependent fashion (Fig. 3). No statistical difference in the RER of *Epac* was noted when cells were treated with either 1 or 10 ng/ml of JH III. A 2-fold increase in the RER of *Epac* was observed when cells were exposed to 100 ng/ml (~0.4 μM) of JH III and the response further increased with doses of 250 ng/ml (~1 μM) and 500 ng/ml (~2 μM). We next examined the temporal response of the RER of *Epac* in relation to a JH challenge. The RER of *Epac* mRNA doubled within 2 h of treatment with 250 ng/ml JH and reached its peak at 4 h (Fig. 4).

3.5. Specificity of the RER of Epac response

To further determine the specificity of the regulation, cells were treated for 4 h with 250 ng/ml MLA, MLEA, (10*R*,11*S*) JH I, (10*R*) JH III, (10*S*) JH III, (10*R*) JH III acid, methyl farnesoate, JHB_3 and methoprene. The RER of *Epac* displayed a rapid and specific rise when challenged with JH homologs and agonists while (10*R*) JH III acid, the major metabolite of JH, elicited no response (Fig. 5). The (10*S*) enantiomer of JH III, which is considered the biologically inactive form in vivo (Goodman et al., 1978), induced a ~2.5-fold increase in the RER of *Epac*; however, the RER of *Epac* when cells were treated with (10*R*) JH III is significantly higher than when they were treated with the (10*S*) JH III enantiomer (Fig. 5). Another compound not expected to have activity was methyl farnesoate; however, it induced a ~2-fold increase in the RER of *Epac*.

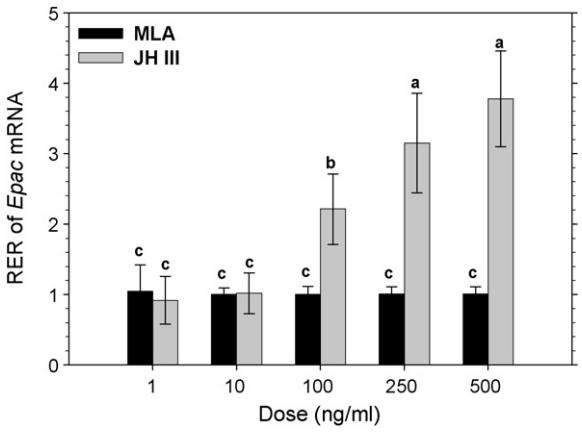

Fig. 3. Dose-dependent response of *Epac* to JH III. S2 cells (5×10^5/ml) were seeded into 60 mm × 15 mm dishes containing 3 ml of medium and allowed to grow for 36 h. Cells were then challenged (4 h) with various doses of (10*R*) JH III or MLA using 1% BSA as a carrier. Real-time qRT-PCR results were generated from the mean RER of *Epac* using two reference gene transcripts (FBGN0002626 and FBGN0034356) compared to MLA as the control. Statistical analysis was performed by one-way ANOVA (LSD). RER means with different letters are significantly different ($p < 0.05$). Error bars represent standard deviation ($n = 6$).

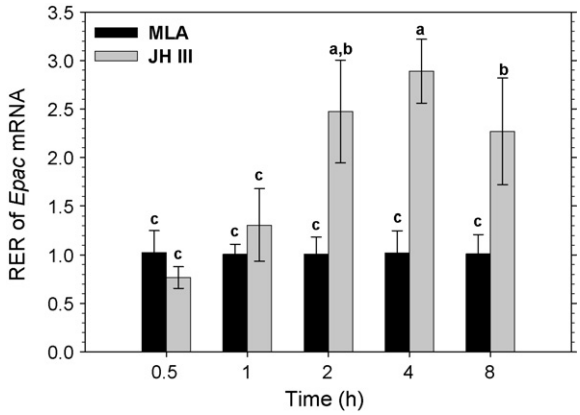

Fig. 4. Time-course of *Epac* response to JH. S2 cells (5×10^5/ml) were seeded into 60 mm × 15 mm dishes containing 3 ml of medium and allowed to grow for 36 h. Cells were then challenged for various times with (10*R*) JH III or MLA (250 ng/ml) using 1% BSA as a carrier. Real-time qRT-PCR results generated the mean RER of *Epac* for two internal standard reference genes (FBGN0002626 and FBGN0034356) using MLA as the control. Results were analyzed by one-way ANOVA (LSD). RER means with different letters are significantly different ($p < 0.05$). Error bars represent standard deviation ($n = 6$).

3.6. Effect of 20-hydroxyecdysone on the expression of Epac

Since JH and ecdysteroids are key regulators of insect development and reproduction, cross-talk between their molecular pathways may be expected (Dubrovsky et al., 2004; Siaussat et al., 2004). We examined the possibility that ecdysteroids affect the RER of *Epac*. Fig. 6 shows that application of 5 μM 20E to S2 cells does not increase the RER of *Epac*; however, JH in the presence of 5 μM 20E induces a significant increase in the RER of *Epac* over JH alone. This response appears dose-dependent, as a challenge of JH plus 10 μM 20E is not significantly different from a challenge of JH alone.

3.7. Expression of Epac in JH-challenged HEK-293 cells

JH agonists such as methoprene have long been used as growth regulators for pest insect populations (Cusson and Palli, 2000). Since there is homology between human and *Drosophila Epac*, the molecular actions of (10*R*) JH III and methoprene on HEK-293 cells

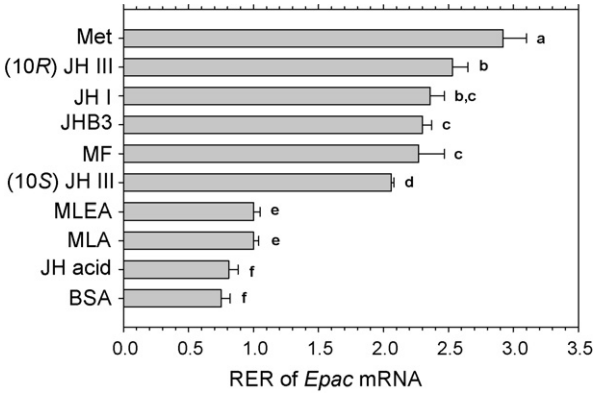

Fig. 5. Specificity of *Epac* expression. S2 cells (5×10^5/ml) were seeded into 60 mm dishes containing 3 ml of medium and allowed to grow for 36 h. Cells were then challenged with various lipids at a concentration of 250 ng/ml for 4 h using 1% BSA as a carrier. Real-time qRT-PCR results were compared by one-way ANOVA (LSD) using the MLA treatment as the control and FBGN0002626 as the internal reference gene. Compounds tested were bovine serum albumin (BSA, control), methyl linoleate (MLA), methyl linolelaidate (MLEA), JH III acid, methyl farnesoate (MF), (10*S*) JH III, (10*R*) JH III, (10*R*,11*S*) JH I (JH I), bisepoxy JH III (JHB_3), and methoprene (Met). RER means with different letters are significantly different ($p < 0.05$). Error bars represent standard deviation ($n = 3$).

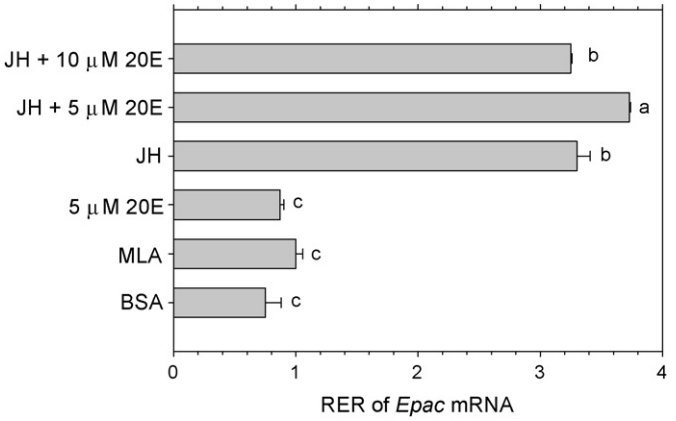

Fig. 6. Effect of JH III and 20-hydroxyecdysone on *Epac* expression. S2 cells (5×10^5/ml) were seeded into 60 mm dishes containing 3 ml of medium and allowed to grow for 36 h. Cells were then challenged with (10*R*) JH III and/or 20-hydroxyecdysone (20E) for 4 h using 1% BSA as a carrier. Real-time qRT-PCR results were compared by one-way ANOVA (LSD) using the MLA treatment as the control and FBGN0002626 as the internal reference gene. RER means with different letters are significantly different ($p < 0.05$). Error bars represent standard deviation ($n = 3$).

were investigated. Total RNA was extracted and real-time qRT-PCR performed to determine whether *Epac* mRNA levels increased after cell exposure to JH or methoprene. Neither (10*R*) JH III nor methoprene affected the mRNA expression level of *Epac* in HEK-293 cells (Fig. 7).

3.8. Expression of Epac in vivo

Tissue origin and the developmental stage at which the primary cells were established, as well as the number of in vitro passages the cells have undergone, have a direct bearing on how the cells will respond to hormone. To test whether *Epac* expression in vivo responded to exogenous JH, mid-third stadium larvae were fed a diet to which methoprene (500 ng/g diet) had been incorporated. Adapting the feeding/hormone introduction protocol of Wilson and Fabian (1986), we found that whole body expression of *Epac* was significantly higher in insects fed methoprene (Table 3). The degree change in *Epac* expression was lower than observed in S2 cells and may be the result of hormone insensitivity in different cell types.

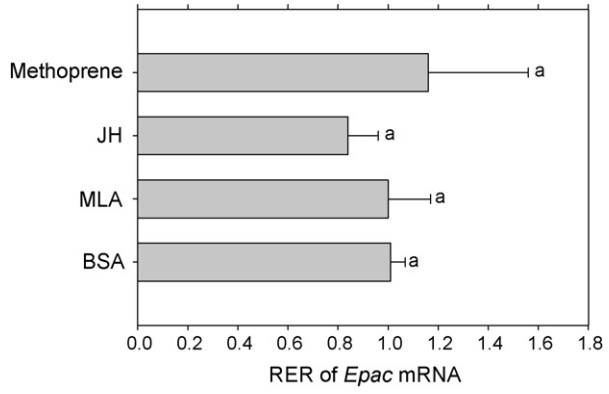

Fig. 7. Effect of JH and methoprene on *Epac* expression in HEK-293 cells. Human embryonic kidney cells (HEK-293) were grown to approximately 70% confluency and then challenged with (10*R*) JH III, methoprene, or MLA for 4 h using 1% BSA as a carrier. Human β-actin (NM_001101) mRNA was used as the internal standard reference gene and MLA was used as the control. The RER means were not significantly different ($p < 0.05$) by one-way ANOVA (LSD). Error bars represent standard deviation ($n = 3$).

Table 3
Relative expression ratios of *Epac* in vivo[a].

	Hours of exposure[b,c]		
Compound	6	12	18
Methoprene[d]	1.18 ± 0.56	1.72 ± 0.99*	1.64 ± 0.61*
Ethanol[e]	1.00 ± 0.62	1.00 ± 0.64	1.00 ± 0.41

[a] Mid-third instars (96 ± 2 h) exposed to methoprene or ethanol for varying periods.
[b] RNA extracted from 10 larvae and used for qRT-PCR.
[c] All RERs are the mean of three determinations; NM_170461, NM_137495, and NM_130639 were used as internal standard references.
[d] Methoprene in ethanol (25 μl) mixed into diet to yield 500 ng of methoprene per g of diet.
[e] Ethanol (25 μl) mixed into diet served as a control.
* Mean RER of *Epac* with methoprene treatment is significantly different from ethanol control as compared by one-way ANOVA (LSD; $p < 0.05$).

4. Discussion

The presence or absence of JH at times outside narrowly prescribed windows during insect development leads to derailment of insect morphogenesis and reproduction (Nijhout, 1994). While these morphological aberrations have been better studied in other insect species, several are known for adult *Drosophila*, including aberrations in bristle formation, rotation of male genitalia and vitellogenesis (Wilson and Fabian, 1986; Riddiford and Ashburner, 1991). Unfortunately, the highly complex mechanisms that give rise to these phenotypic responses are difficult to study at the molecular level. Using an in vitro approach, several genes have been identified as targets of JH action in *Drosophila* cells of embryonic origin (Dubrovsky et al., 2004; Kethidi et al., 2006; Li et al., 2007; Willis et al., in press). The present work, which combines both in vitro and in vivo studies, builds on our initial observation that several genes in *Drosophila* S2 cells are regulated by JH III (Willis et al., in press).

Using cDNA microarray technology, we discovered that JH significantly altered the abundance of *Epac* (Willis et al., in press). Epac is activated in the presence of elevated cAMP and in turn activates Rap1, a low molecular weight GTPase of the Ras family (Bos, 2005). Rap1 cycles between an inactive GDP-bound form and an active GTP-bound form and is aided in the recycling process by Epac. In vertebrate cells, Epac activation of Rap1 leads to integrin-mediated cell adhesion (Rangarajan et al., 2003), stabilization of cytoskeletal–integrin connections (Lyle et al., 2008), formation of vascular endothelial cell barriers (Fukuhara et al., 2005; Kooistra et al., 2005), formation of cardiac gap junctions (Somekawa et al., 2005), increase in mitogen-activated protein kinase activity and signaling (Wang et al., 2006) and interaction with PKA in thyroid-stimulating hormone mediated cell proliferation (Hochbaum et al., 2008).

The introduction of labile lipophilic compounds such as JH to an in vitro culture system poses several problems. In the intact insect, esterases and epoxide hydrolase are responsible for converting the hormone to more polar, inactive metabolites (see Goodman and Granger, 2005 for a review). Because S2 cells secrete hydrolytic enzymes into the medium, we attempted to block JH-specific esterase using a potent inhibitor, OTFP (Abdel-Aal and Hammock, 1985); however, this was only partially successful as virtually all of the hormone was metabolized within the 4 h incubation period. This may be due to the presence of OTFP-insensitive esterases that metabolize the hormone. This could explain why larger than expected doses of JH III are required to initiate the rise in the RER of *Epac*. In addition to its lability, JH is surface-active and binds nonspecifically to hydrophobic surfaces (Kramer et al., 1976). To overcome both nonspecific binding and degradation, we initially used 0.1% BSA as a carrier molecule (Willis et al., in press). In the present study, we found that 1% BSA was optimal for maximal

induction of *Epac*. BSA contains multiple hydrophobic binding sites (Westphal, 1986) and thus serves as a hormone reservoir, reducing degradation and nonspecific binding.

One of the striking features of JH biochemistry is the number of compounds that have biological activity (Slama et al., 1974). The search for these agonists has a practical side as they may be used as insect pest control agents (Cusson and Palli, 2000) and are especially attractive as they have no biological activity in vertebrates (Henrick et al., 1973). Yet, because Epac is an important component in Rap1 activation in vertebrates (Schmidt et al., 2001; Keiper et al., 2004; López De Jesús et al., 2006), there remains the risk that the synthetic agonists used as insect growth regulators may have undiscovered long-term deleterious effects in vertebrates. That concern is mitigated as both (10*R*) JH III and methoprene, the most active compounds on *Drosophila* S2 *Epac*, had no effect on the RER of *Epac* in at least one human cell line, HEK-293 (Fig. 7).

A potent insect growth regulator, methoprene, which lacks an O-methyl ester and an epoxide moiety, was the most active of the compounds tested and was significantly different from (10*R*) JH III in initiating the increase in the RER of *Epac* (Fig. 5). JHB_3, a product of the ring gland in *Drosophila* and other cyclorrhaphous dipteran larvae (Richard et al., 1989 and see Goodman and Granger, 2005 for a review), proved active in S2 cells (Fig. 5); its high levels during the mid-third stadium of the fly (Jones and Jones, 2007) further supports the contention that JHB_3 is a juvenile hormone unique to the more evolutionarily derived dipterans. Methyl farnesoate, a precursor to JH III which lacks the epoxide moiety, also displayed moderate activity in increasing the RER of *Epac* (Fig. 5). Like JHB_3, methyl farnesoate has been found at surprisingly high levels in *Drosophila* mid-third instars (Jones and Jones, 2007). Methyl farnesoate is also present in the embryos of several insect species but its role is still unclear (Cusson et al., 1991 and see Goodman and Granger, 2005 for a review). The activity of the (10*S*) enantiomer, normally thought of as biologically inactive, proved surprising (Fig. 5). While its activity was significantly lower than that of (10*R*) JH III, it was nevertheless more active than controls. As expected, JH acid, the product of esterase cleavage at the C1 position of the hormone, was inactive in this system. It is important to note that 20E cannot induce a substantial rise in the RER of *Epac* (Fig. 6), which makes this model easier to dissect.

We explored only a few of the many chemically diverse compounds that have JH-like activity (Davey, 2000); it may well be that the high degree of cross-talk between signal transduction pathways (see Gerits et al., 2008 for a review) allows such diverse compounds as peptides, thyroid hormones, and sesquiterpenoids to display JH-like activity (Wheeler and Nijhout, 2003; Goodman and Granger, 2005).

The relationship between JH, Rap1 and *Epac* presents an intriguing glimpse into how JH may act at the molecular level. In *Drosophila*, cellular functions associated with Rap1 include cellular architecture, mobility and cell adhesion (Asha et al., 1999; Knox and Brown, 2002). In wild-type embryos, the nuclei of cells surrounding the ventral furrow in gastrulating embryos are apically located but move basally as the cells initiate apical constriction to form the ventral furrow. In Rap1 mutants, the nuclei in cells of the ventral furrow are not uniform in their distribution and fail to migrate basally as the ventral furrow forms; moreover, cells in this region display aberrant shapes that ultimately lead to abnormal gastrulation (Asha et al., 1999).

In wing disc cells mutant for Rap1, the cells display abnormal migration, suggesting that loss of Rap1 function disrupts normal cell–cell adhesion that maintains lineage-related cells in coherent groups (Knox and Brown, 2002). Wild-type cells with functioning Rap1 display an even distribution of adherens junctions in their apical region; cells mutant for Rap1 apparently condense their adherens junctions to one side of the cell, disrupting cell migration and attachments. Thus, activation of the Epac–Rap1 pathway leads to a maintenance of junctional integrity while lack of activated Rap1 or Epac leads to loss of structural integrity (Fukuhara et al., 2005; Kooistra et al., 2005; Wittchen et al., 2005). The effects of activating the Rap1–Epac pathway are consistent with the known morphogenetic action of JH in insects (Truman and Riddiford, 2007).

If the JH-induced increase in the RER of *Epac* ultimately leads to an increase in the gene product within the target cell, the Rap1–Epac pathway would be activated given a concomitant rise in cAMP. The agent responsible for a rise in cAMP might be JH, as seen in ovarian tissue (Pszczolkowski et al., 2005). A JH-induced rise in cAMP would activate PKA, which dissociates into catalytic and regulatory subunits that migrate into the nucleus to activate various transcription factors such as cAMP-response element binding protein (CREB) (Sands and Palmer, 2008). In addition to up-regulating Epac abundance, elevated cAMP levels would activate Epac and the Rap1 signal transduction pathway. While cAMP titers have yet to be determined in this system, computational analysis of the *Drosophila Epac* upstream region does indicate a number of conserved CREB response elements (Sassone-Corsi, 1995; Zhang et al., 2005; Sands and Palmer, 2008). In addition to the CREB response elements in the upstream region, three regions with partial homology to *Drosophila* JH response elements (Li et al., 2007) are located in the upstream region and within the first intron, all homologous regions lying within 2.5 kb of the start site. Embedded within *Epac* between the second and third exons lies Cytochrome P450-6a2 (*CYP6a2*), whose gene product is involved in insecticide and toxic plant secondary compound metabolism (Bhaskara et al., 2006). JH did not increase the RER of *CYP6a2* to detectable levels (data not shown) despite the fact that its coding and regulatory regions are entirely encompassed by *Epac*. Interestingly, *CYP6a2* in S2 cells is up-regulated by cAMP and caffeine, an inhibitor of phosphodiesterase, which metabolizes cAMP (Bhaskara et al., 2008).

Epac provides a particularly attractive target for studying the molecular action of JH. In the present study, we have demonstrated that JH, in the absence of 20E, alters the expression of this important cAMP-sensing molecule. The up-regulation of *Epac* in response to elevated hormone or agonist levels can be observed both in vitro and in vivo. The in vitro response is rapid and the Rap1–Epac signalling pathway putatively activated by JH is consistent with its morphostatic actions. The identification of a well-studied signalling pathway that is activated by JH should provide a robust experimental model to better understand the molecular action of this important developmental hormone.

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References

Abdel-Aal, Y.A.I., Hammock, B.D., 1985. 3-Octylthio-1,1,1-trifluoro-2-propanone, a high affinity and slow binding inhibitor of juvenile hormone esterase from *Trichoplusia ni* (hübner). Insect Biochem. 15, 111–122.

Asha, H., de Ruiter, N., Wang, M., Hariharan, I., 1999. The Rap1 GTPase functions as a regulator of morphogenesis in vivo. EMBO J. 18, 605–615.

Belles, X., 2004. Vitellogenesis directed by juvenile hormone. In: Raikhel, A., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates. Science Publishers, Enfield, NH, pp. 157–198.

Bhaskara, S., Chandrasekharan, M., Ganguly, R., 2008. Caffeine induction of Cyp6a2 and Cyp6a8 genes of *Drosophila melanogaster* is modulated by cAMP and D-JUN protein levels. Gene 415, 49–59.

Bhaskara, S., Dean, E., Lam, V., Ganguly, R., 2006. Induction of two cytochrome P450 genes, Cyp6a2 and Cyp6a8, of *Drosophila melanogaster* by caffeine in adult flies and in cell culture. Gene 377, 56–64.

Bos, J., 2005. Linking Rap to cell adhesion. Curr. Opin. Cell Biol. 17, 123–128.
Bos, J., 2006. Epac proteins: multi-purpose cAMP targets. Trends Biochem. Sci. 31, 680–686.
Chang, E., Coudron, T., Bruce, M., Sage, B., O'connor, J., Law, J., 1980. Juvenile hormone-binding protein from the cytosol of *Drosophila* Kc cells. Proc. Natl. Acad. Sci. U.S.A. 77, 4657–4661.
Cusson, M., Miller, D., Goodman, W., 1997. Characterization of antibody 444 using chromatographically purified enantiomers of juvenile hormones I, II, and III: implications for radioimmunoassays. Anal. Biochem. 249, 83–87.
Cusson, M., Palli, S., 2000. Can juvenile hormone research help rejuvenate integrated pest management? Can. Entomol. 132, 263–280.
Cusson, M., Yagi, K.J.A., Ding, Q.A., Duve, H.A., Thorpe, A.A., McNeil, J.N.A., Tobe, S.S., 1991. Biosynthesis and release of juvenile hormone and its precursors in insects and crustaceans: the search for a unifying arthropod endocrinology. Insect Biochem. 21, 1–6.
Davey, K., 2000. The modes of action of juvenile hormones: some questions we ought to ask. Insect Biochem. Mol. Biol. 30, 663–669.
de Rooij, J., Zwartkruis, F., Verheijen, M., Cool, R., Nijman, S., Wittinghofer, A., Bos, J., 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396, 474–477.
Delany, A., 2001. Measuring transcription of metalloproteinase genes. Nuclear run-off assay vs. analysis of hnRNA. Methods Mol. Biol. 151, 321–333.
Dubrovsky, E., Dubrovskaya, V., Berger, E., 2004. Hormonal regulation and functional role of Drosophila E75A orphan nuclear receptor in the juvenile hormone signaling pathway. Dev. Biol. 268, 258–270.
Echalier, G., 1997. Drosophila Cells in Culture. Academic Press, San Diego, CA.
Elferink, C., Reiners, J.J., 1996. Quantitative RT-PCR on CYP1A1 heterogeneous nuclear RNA: a surrogate for the in vitro transcription run-on assay. Biotechniques 20, 470–477.
Flatt, T., Tu, M., Tatar, M., 2005. Hormonal pleiotropy and the juvenile hormone regulation of *Drosophila* development and life history. Bioessays 27, 999–1010.
Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K., Mochizuki, N., 2005. Cyclic AMP potentiates vascular endothelial cadherin-mediated cell–cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. Mol. Cell. Biol. 25, 136–146.
Gerits, N., Kostenko, S., Shiryaev, A., Johannessen, M., Moens, U., 2008. Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: comradeship and hostility. Cell. Signal. 20, 1592–1607.
Giese, C., Spindler, K., Emmerich, H., 1977. The solubility of insect juvenile hormone in aqueous solutions and its adsorption by glassware and plastics. Z. Naturforsch. 32, 158–160.
Goodman, W., Schooley, D., Gilbert, L., 1978. Specificity of the juvenile hormone binding prote. The geometrical isomers of juvenile hormone I. Proc. Natl. Acad. Sci. U.S.A. 75, 185–189.
Goodman, W.G., Adams, B., 1984. Semipreparative synthesis and purification of juvenile hormone acids by high-performance liquid chromatography. J. Chromatogr. 294, 447–451.
Goodman, W.G., Granger, N.A., 2005. The juvenile hormones. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science. Elsevier, Amsterdam, pp. 319–408.
Henrick, C., Staal, G., Siddall, J., 1973. Alkyl 3,7,11-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. J. Agric. Food Chem. 21, 354–359.
Hochbaum, D., Hong, K., Barila, G., Ribeiro-Neto, F., Altschuler, D., 2008. Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis. J. Biol. Chem. 283, 4464–4468.
Jahn, C., Charkowski, A., Willis, D., 2008. Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. J. Microbiol. Methods 75, 318–324.
Jones, D., Jones, G., 2007. Farnesoid secretions of dipteran ring glands: what we do know and what we can know. Insect Biochem. Mol. Biol. 37, 771–798.
Jones, G., 1995. Molecular mechanisms of action of juvenile hormone. Annu. Rev. Entomol. 40, 147–169.
Jones, G., Sharp, P., 1997. Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. Proc. Natl. Acad. Sci. U.S.A. 94, 13499–13503.
Keiper, M., Stope, M., Szatkowski, D., Böhm, A., Tysack, K., Vom Dorp, F., Saur, O., Oude Weernink, P., Evellin, S., Jakobs, K., Schmidt, M., 2004. Epac- and Ca^{2+}-controlled activation of Ras and extracellular signal-regulated kinases by Gs-coupled receptors. J. Biol. Chem. 279, 46497–46508.
Kethidi, D., Li, Y., Palli, S., 2006. Protein kinase C mediated phosphorylation blocks juvenile hormone action. Mol. Cell. Endocrinol. 247, 127–134.
Klages, G., Emmerich, H., Peter, M.G., 1980. High-affinity binding sites for juvenile hormone I in the larval integument of *Drosophila hydei*. Nature 286, 282–285.
Knox, A., Brown, N., 2002. Rap1 GTPase regulation of adherens junction positioning and cell adhesion. Science 295, 1285–1288.
Kooistra, M., Corada, M., Dejana, E., Bos, J., 2005. Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett. 579, 4966–4972.
Kramer, K., Dunn, P., Peterson, R., Law, J., 1976. Interaction of juvenile hormone with binding proteins in insect hemolymph. In: LI, G. (Ed.), The Juvenile Hormones. Plenum Press, New York, pp. 327–341.
Li, Y., Zhang, Z., Robinson, G., Palli, S., 2007. Identification and characterization of a juvenile hormone response element and its binding proteins. J. Biol. Chem. 282, 37605–37617.
Lipson, K., Baserga, R., 1989. Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe. Proc. Natl. Acad. Sci. U.S.A. 86, 9774–9777.
Lyle, K., Raaijmakers, J., Bruinsma, W., Bos, J., de Rooij, J., 2008. cAMP-induced Epac-Rap activation inhibits epithelial cell migration by modulating focal adhesion and leading edge dynamics. Cell. Signal. 20, 1104–1116.
López De Jesús, M., Stope, M., Oude Weernink, P., Mahlke, Y., Börgermann, C., Ananaba, V., Rimmbach, C., Rosskopf, D., Michel, M., Jakobs, K., Schmidt, M., 2006. Cyclic AMP-dependent and Epac-mediated activation of R-Ras by G protein-coupled receptors leads to phospholipase D stimulation. J. Biol. Chem. 281, 21837–21847.
Nijhout, H., 1994. Insect Hormones. Princeton University Press, Princeton, NJ.
Palli, S., Osir, E., Eng, W., Boehm, M., Edwards, M., Kulcsar, P., Ujvary, I., Hiruma, K., Prestwich, G., Riddiford, L., 1990. Juvenile hormone receptors in insect larval epidermis: identification by photoaffinity labeling. Proc. Natl. Acad. Sci. U.S.A. 87, 796–800.
Pfaffl, M., Tichopad, A., Prgomet, C., Neuvians, T., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515.
Pszczolkowski, M., Peterson, A., Srinivasan, A., Ramaswamy, S., 2005. Pharmacological analysis of ovarial patency in *Heliothis virescens*. J. Insect Physiol. 51, 445–453.
Rangarajan, S., Enserink, J., Kuiperij, H., de Rooij, J., Price, L., Schwede, F., Bos, J., 2003. Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. J. Cell Biol. 160, 487–493.
Rasmussen, R., 2001. Quantification on the LightCycler instrument. In: Meurer, S., Wittwer, C., Nakagawara, K. (Eds.), Rapid Cycle Real-Time PCR: Methods and Applications. Springer-Verlag, Berlin, pp. 21–34.
Richard, D., Applebaum, S., Sliter, T., Baker, F., Schooley, D., Reuter, C., Henrich, V., Gilbert, L., 1989. Juvenile hormone bisepoxide biosynthesis *in vitro* by the ring gland of *Drosophila melanogaster*: a putative juvenile hormone in the higher Diptera. Proc. Natl. Acad. Sci. U.S.A. 86, 1421–1425.
Riddiford, L., 1996. Juvenile hormone: the status of its "status quo" action. Arch. Insect Biochem. Physiol. 32, 271–286.
Riddiford, L., 2008. Juvenile hormone action: a 2007 perspective. J. Insect Physiol. 54, 895–901.
Riddiford, L., Ashburner, M., 1991. Effects of juvenile hormone mimics on larval development and metamorphosis of *Drosophila melanogaster*. Gen. Comp. Endocrinol. 82, 172–183.
Rotenberg, D., Thompson, T., German, T., Willis, D., 2006. Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. J. Virol. Methods 138, 49–59.
Sands, W., Palmer, T., 2008. Regulating gene transcription in response to cyclic AMP elevation. Cell. Signal. 20, 460–466.
Sassone-Corsi, P., 1995. Transcription factors responsive to cAMP. Annu. Rev. Cell Dev. Biol. 11, 355–377.
Schmidt, M., Evellin, S., Weernink, P., von Dorp, F., Rehmann, H., Lomasney, J., Jakobs, K., 2001. A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. Nat. Cell Biol. 3, 1020–1024.
Schooley, D., Bergot, B., Dunham, L., Siddall, J., 1975. Enviromental degradation of the insect growth regulator methoprene (isopropyl (2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate). II. Metabolism by aquatic microorganisms. J. Agric. Food Chem. 23, 293–298.
Sevala, V., Davey, K., 1989. Action of juvenile hormone on the follicle cells of *Rhodnius prolixus*—evidence for a novel regulatory mechanism involving protein kinase C. Experentia 45, 355–356.
Shemshedini, L., Lanoue, M., Wilson, T., 1990. Evidence for a juvenile hormone receptor involved in protein synthesis in *Drosophila melanogaster*. J. Biol. Chem. 265, 1913–1918.
Siaussat, D., Bozzolan, F., Queguiner, I., Porcheron, P., Debernard, S., 2004. Effects of juvenile hormone on 20-hydroxyecdysone-inducible EcR, HR3, E75 gene expression in imaginal wing cells of Plodia interpunctella lepidoptera. Eur. J. Biochem. 271, 3017–3027.
Slama, K., Romanuk, M., Sorm, F., 1974. Insect Hormones and Bioanalogues. Springer-Verlag, New York.
Somekawa, S., Fukuhara, S., Nakaoka, Y., Fujita, H., Saito, Y., Mochizuki, N., 2005. Enhanced functional gap junction neoformation by protein kinase A-dependent and Epac-dependent signals downstream of cAMP in cardiac myocytes. Circ. Res. 97, 655–662.
Truman, J., Riddiford, L., 2007. The morphostatic actions of juvenile hormone. Insect Biochem. Mol. Biol. 37, 761–770.
Venkatesh, K., Abdel-Aal, Y., Armstrong, F., Roe, R., 1990. Characterization of affinity-purified juvenile hormone esterase from the plasma of the tobacco hornworm *Manduca sexta*. J. Biol. Chem. 265, 21727–21732.
Wang, Z., Dillon, T., Pokala, V., Mishra, S., Labudda, K., Hunter, B., Stork, P., 2006. Rap1-mediated activation of extracellular signal-regulated kinases by cyclic AMP is dependent on the mode of Rap1 activation. Mol. Cell. Biol. 26, 2130–2145.
Westphal, U., 1986. Steroid-protein interactions II. Monogr. Endocrinol. 27, 1–603.
Wheeler, D., Nijhout, H., 2003. A perspective for understanding the modes of juvenile hormone action as a lipid signaling system. Bioessays 25, 994–1001.
Willis, D., Wang, J., Lindholm, J., Orth, A., Goodman, W., in press. Microarray analysis of juvenile hormone response in *Drosophila melanogaster* S2 cells. J. Insect Sci.
Wilson, T., DeMoor, S., Lei, J., 2003. Juvenile hormone involvement in *Drosophila melanogaster* male reproduction as suggested by the methoprene-tolerant(27) mutant phenotype. Insect Biochem. Mol. Biol. 33, 1167–1175.

Wilson, T., Fabian, J., 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. Dev. Biol. 118, 190–201.
Wisniewski, J., Kochman, M., 1984. Juvenile hormone binding protein from silk gland of *Galleria mellonella*. FEBS Lett. 171, 127–130.
Wisniewski, J., Wawrzenczyk, C., Prestwich, G., Kochman, M., 1988. Juvenile hormone binding proteins from the epidermis of *Galleria mellonella*. Insect Biochem. 18, 29–36.
Wittchen, E., Worthylake, R., Kelly, P., Casey, P., Quilliam, L., Burridge, K., 2005. Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J. Biol. Chem. 280, 11675–11682.
Yamamoto, K., Chadarevian, A., Pellegrini, M., 1988. Juvenile hormone action mediated in male accessory glands of *Drosophila* by calcium and kinase C. Science 239, 916–919.
Zhang, X., Odom, D., Koo, S., Conkright, M., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J., Emerson, B., Hogenesch, J., Unterman, T., Young, R., Montminy, M., 2005. Genome-wide analysis of cAMP response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc. Natl. Acad. Sci. U.S.A. 102, 4459–4464.